

REMARKS

Applicants thank Examiner Leavitt for taking time to hold a telephonic interview on June 30, 2009. Applicants agree with the substance of the Interview Summary provided by the Examiner on July 2, 2009.

The claims

Claims 1-3, 6-8, 11-12, 15-17, 19-21, 23-26, and 28 are pending. Claims 4-5, 9-10, 18, 22, 27, and 29-41 are withdrawn. Claims 13-14 are canceled. Some of the claims are amended to clarify the Markush claim language. The amendments do not add new matter, and do not narrow the scope of the claims.

Applicant requests that, once the claims currently under consideration are allowed, the claims reciting species which were not elected for preliminary examination (*e.g.*, claims 4, 5, 9, 10, 18, 22 and 27) be examined. It is noted that claims 4, 5, 9, 10, 18, 22 and 27 recite additional elements that were not recited in the examined claims. That is, they recite "further comprising" treating the mammalian mesenchymal cells with at least one secondary agent. Therefore, provided that the elected claims are deemed patentable, these additional claims, which merely add additional elements or steps, will not require a further search and examination, and should also be deemed to be patentable.

Obviousness rejections

The Examiner cites the Paralkar patent application ("**Paralkar**") for its alleged disclosure of *in vitro* and *in vivo* methods for enhancing bone formation in mammals, including humans, comprising administering HMG-CoA reductase inhibitors such as statins in combination with prostaglandin agonists. The Examiner points in particular to disclosures in the reference that statins allegedly enhance the expression of mRNA for BMP-2, an agent that has been reported to induce osteogenic differentiation.

Many of the statements that the Examiner attributes to Paralkar were, in fact, the characterization by Paralkar of other publications that are referred to in that patent application. Without discussing the statements in Paralkar in detail, applicant notes that the reference reports that certain statins allegedly enhance the expression of markers associated with bone formation, or stimulate bone formation, presumably via a mechanism of stimulating the

formation of certain BMPs (*e.g.*, BMP-2). However, the reference does not suggest or disclose that oxysterols can enhance osteoblastic differentiation.

The Examiner argues that because both oxysterols and statins have been reported to inhibit HMG-CoA reductase activity, it would have been obvious for a skilled person to substitute oxysterols for the statins used by Paralkar, in order to induce osteoblastic differentiation (in MSCs), with a reasonable expectation of success.

However, there is a logical disconnect to the allegation that oxysterols could substitute for the statins: Paralkar did not demonstrate a causal relationship between the ability of statins to inhibit HMG-CoA reductase activity and their alleged ability to induce osteoblastic differentiation. Thus, even if it were the case that certain statins, which can inhibit HMG-CoA reductase, could induce osteoblastic differentiation in MSCs, this does not necessarily mean that OTHER agents which inhibit HMG-CoA reductase, such as oxysterols, would also induce osteoblastic differentiation in MSCs.

Biological phenomena, such as the physiology of osteoblastic differentiation, are complex and poorly understood. It would have been unpredictable at the time of filing the present application whether two different inhibitors of HMG-CoA reductase would have the same effect on, *e.g.*, osteoblastic differentiation. Absent the establishment of a causal link between the ability of an agent to inhibit HMG-CoA reductase and the ability of the agent to induce osteoblastic differentiation, there would have been no motivation for a skilled worker to substitute an oxysterol for the statins of the Paralkar reference, with the requisite reasonable expectation of success.

In fact, importantly, the inventor and his colleagues reported contemporaneously with the filing of the present application that at least one statin - metavastin (which is one of the statins listed by Paralkar, in paragraph [0011], line 9) - inhibits HMG-CoA reductase, but instead of stimulating osteoblastic differentiation in MSCs, it has the opposite effect and inhibits the induction of osteoblastic differentiation of marrow stromal cells or MSCs; and it inhibits the activity of alkaline phosphatase, which is a marker of osteoblastic differentiation. See, *e.g.*, Parhami *et al* (2002) *J. Bone & Mineral Res* 17, 1997, which is attached hereto, particularly the Abstract and page 1998, left column. The Parhami *et al* (2002) reference, by showing that at least one type of statin fails to stimulate osteoblastic differentiation in MSC, even though it inhibits HMG-CoA reductase, supports applicant's argument there is not a causal relationship

between the ability of an agent to inhibit HMG-CoA reductase and its ability to stimulate osteoblastic differentiation in MSCs and, in fact, teaches away from there being any such causality. It was thus not at all predictable at the time of filing the application whether a particular agent that inhibits HMG-CoA would stimulate or would inhibit osteoblastic differentiation of MSCs. This, alone, is sufficient to counter the allegation that Paralkar suggests or discloses that oxysterols can be substituted for statins to achieve osteogenesis, with a reasonable expectation of success.

The "**Parish**" reference does not remedy the defects of Paralkar.

To address the defect in Paralkar that it does not specifically teach osteoblastic differentiation with an *oxysterol*, the Examiner refers to the Parish paper for its disclosure that side-chain oxysterols are potent inhibitors of HMG-CoA reductase. Parish does not mention osteoblastic differentiation. Even if this paper did disclose that side-chain oxysterols are potent inhibitors of HMG-CoA reductase, the paper does not remedy the defect of Paralkar that it fails to show that an oxysterol (or, for that matter, any particular inhibitor of HMG-CoA reductase) would be expected to induce osteoblastic differentiation. Furthermore, Parish does not suggest or disclose that an inhibitor of HMG-CoA reductase can inhibit adipocyte differentiation of mammalian mesenchymal stem cells (as is required by claim 1).

The "**Wang** " reference does not remedy the defects of Paralkar, or of Paralkar taken with Parish.

The Examiner cites the Wang *et al.* reference for its alleged disclosure that treatment with statins (in this case, lovastatin) inhibits adipocyte differentiation and induces osteoblastic differentiation of mouse MSCs, as indicated by markers such as alkaline phosphatase activity, osteocalcin mRNA and cAMP production.

Like the other references cited by the Examiner, Wang is directed to the effects of a statin, and does not suggest or disclose that an oxysterol could substitute for the statin to, *e.g.*, induce osteoblastic differentiation. For this reason alone, the reference does not remedy the defects of Paralkar, or of Paralkar taken with Parish, which fail to teach that an oxysterol (or, for that matter, any particular inhibitor of HMG-CoA reductase) would be expected to induce osteoblastic differentiation.

Furthermore, and supplementally, the Wang reference does not directly test the ability of a statin - lovastatin - to inhibit adipocyte differentiation and induce osteoblastic differentiation of MSCs. Rather, this paper is directed to the development of a treatment that can preserve bone mass and prevent osteonecrosis in animals that are treated with steroids. That is, the paper studies the ability of the statin to *reverse* the effects of steroids (osteonecrosis, or the death of bone and bone cells) on marrow osteoprogenitor cells. The cells or animals are first treated with steroids, which induce fat expression and inhibit osteoblastic gene expression (or, in the animals, cause bone death, or osteonecrosis). The cells or animals are then treated with the statin to determine if it can reverse those phenomena. Even if Wang showed that statins can reverse the osteonecrotic effects of steroids (death of bone and bone cells), the reference does not show that the statins can induce osteogenic differentiation. For example, if steroids cause osteonecrosis in animals by inducing the death of osteoblasts, then the statins could be reversing this effect by inhibiting osteoblast death, rather than by stimulating osteogenesis.

Therefore, Wang *et al.*, either alone or in combination with Parish, does not remedy the defect of Paralkar, that it fails to suggest or disclose that an oxysterol (or, for that matter, any particular inhibitor of HMG-CoA reductase) would be expected to induce osteoblastic differentiation in an MSC.

For at least the preceding reasons, neither the cited references nor the art at the time the invention was made provide a motivation to combine the cited references to achieve the presently claimed invention, with a reasonable expectation of success, and thus do not render the present claims obvious. Applicant requests that the obviousness rejection be withdrawn.

Obviousness-type double patenting rejections

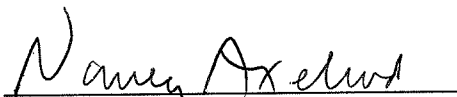
Applicant, without acquiescing to any rejection, respectfully requests that the two provisional double-patenting rejections be held in abeyance until the allowance of claims in the instant application. While in no way admitting that the present claims are obvious over the claims of the cited applications, upon allowance of the claims of the instant application, Applicant will consider filing terminal disclaimers.

In view of the preceding arguments, applicant believed that the present claims are in condition for allowance, which action is respectfully requested.

A one-month Extension of Time accompanies this Reply. Should any additional fee be deemed due, please charge such fee to our Deposit Account No. 22-0261, reference our docket number 58086-241892, and notify the undersigned accordingly

Respectfully submitted,

Date: July 23, 2009

A handwritten signature in cursive script, reading "Nancy Axelrod", is written over a horizontal line.

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Role of the Cholesterol Biosynthetic Pathway in Osteoblastic Differentiation of Marrow Stromal Cells

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ABSTRACT

Cholesterol is an important molecule that plays a key role in regulating cellular differentiation and function. Although the possible role of lipids has been implicated in regulating osteoblastic cells, the role of cholesterol in that process is not well defined. In this study we have examined the role of the cellular cholesterol biosynthetic pathway on osteoblastic differentiation of marrow stromal cells (MSCs). Treatment of pluripotent mouse MSCs M2-10B4 with inhibitors of the cholesterol biosynthetic pathway mevastatin or mevinolin inhibited the maturation of these cells into functional osteoblastic cells. This was determined by the inhibition of the activity and expression of alkaline phosphatase (ALP), a key enzyme involved in differentiation and mineralization of osteoblastic cell cultures, as well as inhibition of mineralization. Mevastatin treatment did not affect expression of the osteoblast-specific gene osteocalcin (OCN). Furthermore, promoter-reporter studies in MSCs showed that mevastatin inhibited activity of the ALP gene promoter, suggesting regulation by derivatives of the cholesterol biosynthetic pathway. The effects of mevastatin and mevinolin were reversed by mevalonate but not by geranylgeraniol or farnesol, intermediates in the cholesterol biosynthetic pathway. Altogether, these results suggest that products of the cholesterol biosynthetic pathway are important for proper development of MSCs into functional osteoblastic cells capable of forming a mineralized matrix. Identification of those molecules may provide new therapeutic approaches to prevent the decline in osteoblastic activity in osteoporosis and aging. (J Bone Miner Res 2002;17:1997-2003)

Key words: cholesterol, osteoblast differentiation, statin, ALP, marrow stromal cells

INTRODUCTION

ALTHOUGH THE LIVER synthesizes most of the body's endogenous cholesterol, most cell types have the capacity for cholesterol biosynthesis.⁽¹⁾ Cholesterol not only serves as a precursor for steroid hormones and bile acids and an essential component of cellular membranes, but together with other intermediates of the cholesterol biosynthetic pathway regulates signaling molecules including hedgehog, Ras, Rab, and Rho.⁽²⁻⁴⁾ The body's total chole-

sterol is derived through the cellular cholesterol biosynthetic pathway and through absorption from the diet.⁽⁴⁾ However, the synthesis and use of cholesterol must be tightly regulated to prevent hyperlipidemia and abnormal deposition of cholesterol within tissues. Human and animal studies have found hypercholesterolemia and the resulting deposition and oxidation of lipids in tissues to lead to atherosclerosis⁽⁵⁾ and relate to carcinogenesis⁽¹⁾ and osteoporotic bone loss.⁽⁶⁾

Bone turnover is regulated by osteoblasts and osteoclasts that produce and resorb bone, respectively. Impaired bone turnover, resulting from increased osteoclastic bone resorption and decreased osteoblastic bone formation, has adverse

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effects on bone quality and quantity in adult organisms and leads to osteoporosis.⁽⁷⁾ Most currently approved therapeutic interventions for osteoporosis block osteoclastic bone resorption. However, because reduced osteoblastic bone formation also contributes to the lowering of bone density in osteoporosis, anabolic agents that target osteoblasts may also be effective in reversing or preventing osteoporotic bone loss. Understanding the mechanisms and factors that regulate the differentiation and activity of osteoblastic cells is fundamental to identification of such therapeutic targets.

Although exogenous lipids have been implicated in the regulation of osteoblastic differentiation,^(8,9) the role of cell-synthesized cholesterol in that process is not clear. Recent studies have indicated that interruption of the cholesterol biosynthetic pathway in osteoblastic cells by HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors may induce differentiation and bone-forming activity in those cells.^(10,11) In contrast, preliminary studies by Nuckolls et al. indicate that one of these inhibitors, lovastatin, reduces endochondral ossification in a cranial base organ culture system.⁽¹²⁾ Although a number of *in vivo* studies in animals and humans have suggested that inhibition of the cholesterol biosynthetic pathway by these inhibitors may increase bone density and reduce fracture risk,^(10,13,14) others have found no effect.⁽¹⁵⁾

To assess the role of cholesterol and its biosynthetic pathway in regulating differentiation and activity of osteoblastic cells, we examined the effect of two inhibitors of cellular cholesterol biosynthesis, mevastatin (compactin) and mevinolin (lovastatin), in regulating osteoblastic differentiation of pluripotent M2-10B4 mouse marrow stromal cells (MSCs) *in vitro*. These agents inhibited HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis,^(16,17) and significantly suppressed the expression and activity of ALP, a key enzyme involved in differentiation and mineralization of osteoblastic cells.⁽¹⁸⁾ They also inhibited ultimate calcium mineral deposition in MSC cultures. Expression of osteocalcin (OC), an osteoblast-specific protein, was not affected by HMG-CoA reductase inhibitor treatment. These results suggest that products of the cholesterol biosynthetic pathway are essential for maturation and mineralization of MSCs.

MATERIALS AND METHODS

Materials

⁴⁵CaCl₂ was purchased from Amersham Corp. (Piscataway, NJ, USA), ascorbate, β -glycerophosphate (β GP), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, thiazoyl blue), mevalonic acid, geranylgeraniol, and farnesol were from Sigma (St. Louis, MO, USA), and RPMI-1640 and α -MEM were from Irvine Scientific (Santa Ana, CA, USA). Mevastatin and mevinolin were purchased from BIOMOL Research Labs (Plymouth Meeting, PA, USA). Luciferase assay system and lysis buffer were obtained from Promega (Madison, WI, USA). The promoter construct containing the mouse liver-bone-kidney isozyme of ALP gene promoter was kindly provided by Dr. T. Kobayashi (Japan). This ALP 1A 5' promoter

fragment (-1832 to +82) was subcloned into the pGL3 basic luciferase reporter vector (Promega).⁽¹⁹⁾

Cell culture

The M2-10B4 mouse MSC line obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) was derived from bone MSCs of a (C57BL/6J \times C3H/HeJ) F1 mouse and supports human and murine myelopoiesis in long-term cultures (ATCC). These cells were cultured in growth medium consisting of RPMI containing 10% heat-inactivated FBS (Hyclone, Logan, UT, USA), and supplemented with 1 mM of sodium pyruvate, 100 U/ml of penicillin, and 100 U/ml of streptomycin (all from Irvine Scientific). To induce osteoblastic differentiation, the cells were incubated in osteogenic medium consisting of the growth medium to which 50 μ g/ml of ascorbate and 3 mM of β GP were added as previously described.⁽²⁰⁾ Primary human MSCs were obtained from BioWhittaker, Inc. (Walkersville, MD, USA) and cultured according to manufacturer's instructions.

ALP activity assay

M2-10B4 cells, seeded in 24-well plates, were treated at 80% confluence with DMSO vehicle alone or test agents in osteogenic media as described previously. Colorimetric ALP activity assay on whole cell extracts was performed as previously described.⁽²⁰⁾ ALP activity was normalized to total protein concentration as assessed by the Bradford assay (Bio-Rad, Hercules, CA, USA).

MTT assay

The MTT assay was used to measure cell viability. Cells were seeded in 24-well plates and treated in the same manner as in the experiments for ALP activity measurements. The MTT assay was performed as previously described.⁽²¹⁾ The data were obtained from quadruplicate wells and assessed as a percentage of control \pm SD.

⁴⁵Ca incorporation assay

Calcification in cell monolayers was quantified by measuring ⁴⁵CaCl₂ incorporation in the extracellular matrix of M2-10B4 cells as previously described.⁽²²⁾

Transient transfection

A standard protocol of Effectene-mediated transfection was followed according to the manufacturer's instructions (Qiagen, San Diego, CA, USA). Briefly, 60–75% confluent M2-10B4 cells in a 12-well plate were overlaid with DNA-Effectene complexes (0.3 μ g of plasmid DNA and 2.4 μ l of 2 mg/ml of Effectene) in α -MEM containing 10% FBS. After transfection, cells were incubated overnight in RPMI containing 10% FBS to allow for recovery. After recovery, the cells were treated for 24 h with vehicle or mevastatin. Cells were lysed and luciferase and β -galactosidase activi-

ties were determined as previously described.⁽²⁰⁾ Luciferase activity was normalized to β -galactosidase activity.

RNA analysis

Cells were plated in 60-mm dishes and total RNA was isolated from confluent monolayers of M2-10B4 cells using the RNA isolation kit from Stratagene according to manufacturer's instructions (Stratagene, La Jolla, CA, USA). RNA (3 μ g) was DNase-treated and reverse-transcribed as previously described. Mouse bone-liver-kidney isozyme of ALP, mouse OC, and GAPDH were amplified using the primers previously described.⁽²³⁾ The amplified polymerase chain reaction (PCR) products were electrophoresed and visualized by autoradiography. Autoradiographs were scanned and semiquantitated with National Institutes of Health (NIH) Image software, version 1.49, public domain program (NIH, Bethesda, MD, USA).⁽²³⁾ ALP and OC band intensities were normalized to GAPDH.

Western blotting and electrophoretic mobility shift assay

Western blot analyses for Cbfa1 and collagen I protein expression and gel mobility shift assay for Cbfa1 were performed as previously described.⁽²³⁾

Statistical analysis

Computer-assisted statistical analyses were performed using the StatView 4.5 program. All *p* values were calculated using ANOVA and Fisher's projected least significant difference (PLSD) significance test. A value of *p* < 0.05 was considered significant.

RESULTS

Effects on markers of osteoblastic differentiation

To test the role of endogenous cholesterol production in osteoblastic differentiation, we treated M2-10B4 MSCs with mevastatin or mevinolin. Both agents caused a significant dose-dependent inhibition of ALP activity compared with untreated control cells (Figs. 1A and 1B). Mevastatin also inhibited ALP activity in primary human MSCs (Fig. 1C), confirming its similar effects in murine and human cells.

Treatment of M2-10B4 cells with 2 μ M of mevastatin for 2, 4, or 6 days showed no change in collagen I or Cbfa-1 protein expression and Cbfa-1 DNA-binding activity as assessed by Western blot analysis and electrophoretic mobility shift assays, respectively (data not shown).

After 9 days of treatment with mevastatin, a dose-dependent inhibition in expression of ALP mRNA but not OC mRNA was observed (Fig. 2). Mineral deposition, the endpoint of osteoblastic differentiation and function, was also assessed by measuring ⁴⁵Ca incorporation into the extracellular matrix of M2-10B4 cells after 14 days. Mevastatin significantly inhibited ⁴⁵Ca incorporation in a dose-

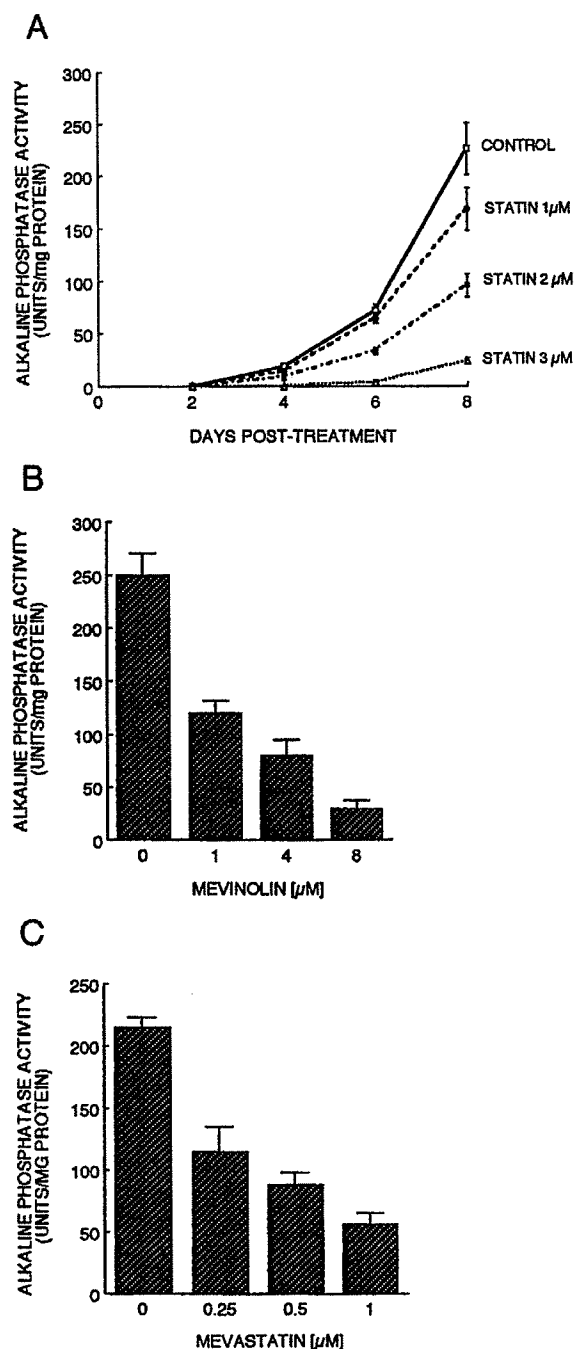


FIG. 1. Effect of mevastatin, mevinolin, and mevastatin on ALP activity in MSCs. M2-10B4 cells at 80% confluence were treated with (A) mevastatin (STATIN) for 2–8 days or (B) mevinolin for 6 days, and (C) primary human MSCs at 80% confluence were treated with mevastatin for 6 days. ALP activity was determined in cell homogenates. Results from a representative of three separate experiments are shown, reported as the mean \pm SD of quadruplicate determinations, normalized to protein concentration (*p* < 0.01 for control vs. mevastatin- or mevinolin-treated cells at all concentrations).

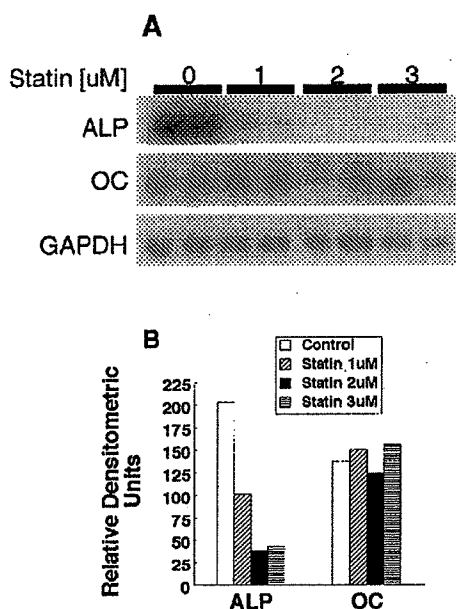


FIG. 2. Effect of mevastatin on gene expression in MSCs. (A) M2-10B4 cells at 80% confluence were treated with mevastatin at the concentrations indicated. After 9 days of incubation, total RNA was isolated from duplicate plates for each condition. Expression of ALP, OC, and GAPDH were detected by semiquantitative reverse transcription (RT)-PCR. A representative of two separate experiments is shown, each lane corresponding to a separate sample; (B) densitometric analysis of the scanned autoradiograph normalized to GAPDH expressed as the mean of duplicate samples.

dependent manner compared with untreated control cells (Fig. 3).

In addition, we examined the effect of mevastatin on cell number and protein levels in M2-10B4 cultures. Treatment for 3 days or 6 days with 1 μ M or 2 μ M of mevastatin did not change the total cell number or protein levels (data not shown) compared with control untreated cells. Treatment with 3 μ M of mevastatin after 6 days consistently resulted in a 10–20% reduction in total cell number (control = $9 \pm 0.15 \times 10^5$; mevastatin = $7.2 \pm 0.2 \times 10^5$ cells/well reported as mean of quadruplicate determinations \pm SD; $p < 0.05$) and protein levels (data not shown). Treatment with 1–3 μ M mevastatin showed no toxicity by morphological criteria and by the MTT cytotoxicity assay (data not shown).

Effect on ALP promoter activity

Because ALP expression is regulated at both transcriptional and posttranscriptional levels, we examined the effect of cholesterol biosynthesis inhibitors on ALP promoter activity. Promoter-reporter studies using the mouse liver/bone/kidney ALP promoter with a luciferase reporter showed a significant 50% inhibition of luciferase activity in the presence of 3 μ M of mevastatin after 24 h, suggesting that mevastatin inhibits ALP at least in part by inhibition of

transcriptional activity (Fig. 4). These effects were not observed when cells were transfected with the pGL3 control vector (data not shown).

Rescue by mevalonic acid

Mevalonic acid, an intermediate in the cholesterol biosynthetic pathway, produced by the reaction catalyzed by HMG-CoA reductase,⁽³⁾ is expected to reverse the effects of HMG-CoA reductase inhibitors in vitro.⁽²⁴⁾ In M2-10B4 cells, mevalonic acid reversed the inhibitory effects of mevastatin and/or mevinolin on ALP activity (Fig. 5), on ALP promoter activity (Fig. 4), and on ⁴⁵Ca incorporation (Fig. 3). In contrast, 10–100 μ M geranylgeraniol and 10–40 μ M farnesol, both downstream side-products of the cholesterol biosynthetic pathway, did not rescue the cells from the inhibitory effect of mevastatin on ALP activity (data not shown). This suggests that the reductase inhibitor effects are not attributable to depletion of these substrates for protein prenylation.

DISCUSSION

These results show a role of the cholesterol biosynthetic pathway in osteoblastic differentiation of MSCs, based on inhibition of differentiation markers by HMG-CoA reductase inhibitors, mevastatin, and mevinolin. These agents inhibited ALP activity and expression, as well as mineralization, without changing Cbfa-1, collagen I, and OC expression. Furthermore, mevastatin inhibited promoter activity of the ALP gene, suggesting that its effect on ALP was at least in part at the transcriptional level. The partial inhibition in ALP promoter activity produced by mevastatin compared with its greater effect on mRNA expression is consistent with the reported regulation of ALP gene expression at both transcriptional and posttranscriptional levels.^(25–27) The lack of effect on Cbfa-1, collagen I, and OC expression suggests that statins suppress mineralization without altering cell identity or lineage. Furthermore, in contrast to Cbfa-1-dependent regulation of collagen I and OC, regulation of ALP expression may be independent of Cbfa-1.⁽¹⁹⁾ Inhibitory effects of mevastatin on ALP but not on Cbfa-1- and Cbfa-1-regulated factors suggests an effect on other regulatory molecules involved in osteoblastic differentiation of MSCs. We speculate that inhibition of ALP is the mechanism for inhibition of mineralization based on evidence that this enzyme is the critical regulator of mineralization of osteoblastic cells.^(28–30) Rescue by mevalonic acid indicates that the mevastatin and mevinolin effects are caused by HMG-CoA reductase inhibition. However, the failure of geranylgeraniol and farnesol to reverse the effects suggests that they are not through inhibition of protein geranylation or farnesylation, an important regulatory mechanism for signaling molecules such as Rab and Rho.⁽⁵⁾ Altogether, these observations support the hypothesis that the cholesterol biosynthetic pathway and its derivatives are essential for maturation and mineralization of MSC cultures.

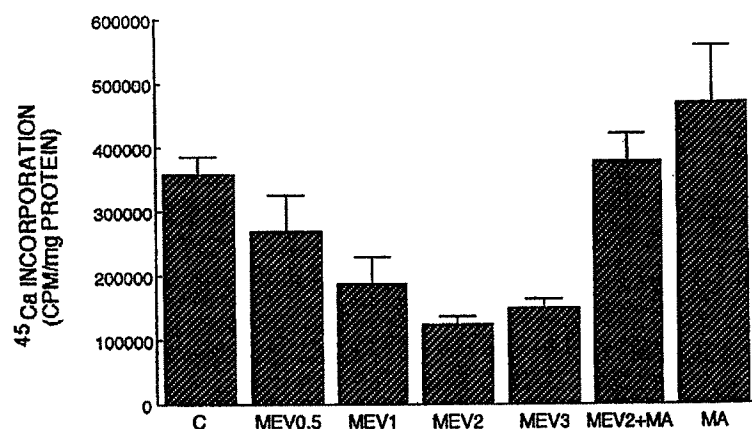


FIG. 3. Effect of mevastatin on mineralization in MSC cultures. M2-10B4 cells at 80% confluence were treated with vehicle (C) or mevastatin (MEV; 0.5–3 μ M) in the presence or absence of mevalonic acid (MA; 1 mM), and 45 Ca incorporation was assessed after 10 days. A representative of two separate experiments is reported as the mean of quadruplicate determinations \pm SD normalized to total protein concentration ($p < 0.01$ for C vs. MEV1–3 μ M and for MEV2 and MEV2 + MA).

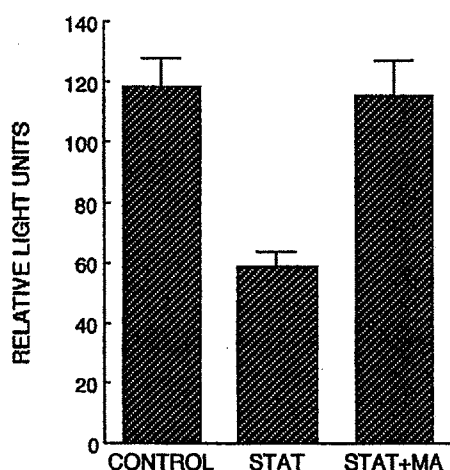


FIG. 4. Effect of mevastatin on ALP promoter activity. M2-10B4 cells were transiently transfected with a construct containing the mouse ALP promoter and luciferase reporter. Control buffer (CONTROL), or 3 μ M of mevastatin (STAT) in the presence or absence of 1 mM of mevalonic acid (MA) were added for 24 h. Cells were then lysed, and luciferase and β -galactosidase activities were determined as previously described. Results from a representative of three separate experiments are reported as the mean relative light units for quadruplicate determinations \pm SD normalized to β -galactosidase activity ($p = 0.007$ for CONTROL vs. STAT and $p = 0.013$ for STAT vs. STAT + MA).

MSCs are pluripotent stem cells that are the precursors of osteoblasts and generate these bone-forming cells during normal bone remodeling and fracture repair.⁽³¹⁾ Several studies have indicated a decrease in the number of MSCs, in parallel with decreased osteoblast number, in aging and osteoporosis.^(32,33) Although the reason for this decline is not clearly understood, a better understanding of factors regulating osteoblastic differentiation of MSCs may help identify new targets such as cholesterol and other products of the cholesterol biosynthetic pathway for intervention in age-related osteoporosis. Previous studies showing that covalent addition of cholesterol to hedgehog is required for its

function,⁽²⁾ which in turn regulates expression of and response to bone morphogenetic proteins (BMPs),^(34,35) further support a role for cholesterol in osteoblastic differentiation.

Some studies have suggested that the statin family of HMG-CoA reductase inhibitors increase bone mineral density (BMD) in mice,⁽¹⁰⁾ in patients with type 2 diabetes mellitus,⁽³⁶⁾ and in postmenopausal women.⁽³⁷⁾ They also decrease the risk of fractures in retrospective nonrandomized studies.^(13,14) One potential mechanism by which statins could exert an anabolic effect is by a direct stimulatory effect on osteoblastic cells.⁽¹⁰⁾ However, because statins are almost entirely cleared in the liver, the expected concentrations at peripheral tissues such as bone would be relatively small.⁽³⁸⁾ An alternative mechanism is the known effect of these agents on hepatocytes, a decrease in plasma cholesterol and resulting decrease in its deposition in peripheral tissues such as artery wall and perhaps bone.^(39,40) Circulating cholesterol can be reduced even without significant effects on the cholesterol biosynthesis by peripheral tissues.⁽⁴⁰⁾ Thus, statins may affect bone indirectly through lipid-lowering. This concept is supported by evidence that hyperlipidemia in mice reduces BMD⁽⁴¹⁾ and that products of lipid oxidation such as minimally oxidized low-density lipoprotein inhibit differentiation and mineralization of osteoblastic cells in vitro and ex vivo.^(20,22) Therefore, although hypercholesterolemia may adversely affect bone, a baseline level of cholesterol synthesis by bone cells may be necessary for their activity and mineralization.

The effects of reductase inhibitors on osteoblastic differentiation vary. Lovastatin and simvastatin at micromolar concentrations promote in vitro osteoblastic differentiation and induce BMP-2 expression in human cells.⁽¹⁰⁾ Simvastatin, at nanomolar concentrations, promotes osteoblastic differentiation of MC3T3-E1 mouse calvarial osteoblastic cells as well as rat marrow stromal.⁽¹¹⁾ On the other hand, Nuckolls et al. report preliminarily that statins inhibit endochondral ossification in a cranial organ culture system.⁽¹²⁾ The cholesterol biosynthetic pathway modulates differentiation and survival of other cell types. Reductase inhibitors and bisphosphonates, which inhibit cholesterol biosynthesis

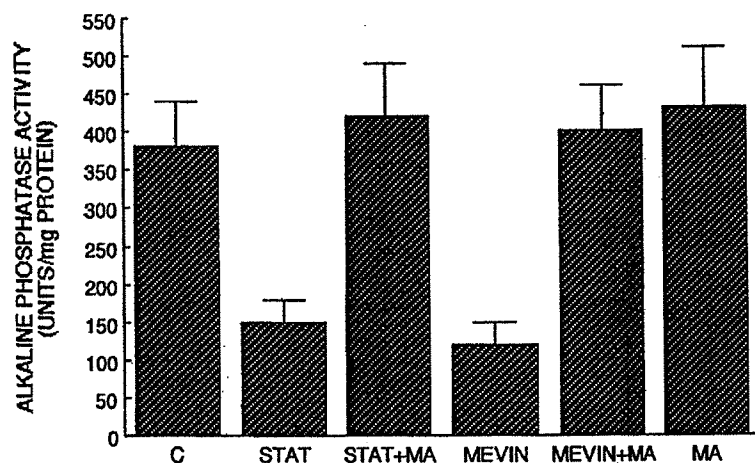


FIG. 5. Effect of mevalonate on inhibition of ALP activity by mevastatin. M2-10B4 cells at 80% confluence were treated with control buffer (C), 2 μ M of mevastatin (STAT), or mevinolin (MEVIN) alone or in combination with 100 μ M of mevalonate (MA). After 6 days, ALP activity was determined in cell homogenates. Results from a representative of three separate experiments are shown, reported as the mean \pm SD of quadruplicate determinations, normalized to protein concentrations ($p < 0.01$ for C vs. STAT or MEVIN, and $p < 0.005$ for STAT and MEVIN alone vs. in combination with MA).

downstream of HMG-CoA reductase, induce apoptosis in macrophages and osteoclasts.⁽⁴²⁾ Products of the cholesterol biosynthetic pathway also mediate the differentiation and growth of enterocytes and epidermal keratinocytes.^(43,44) In vitro findings may vary with culture conditions and cell types. Because HMG-CoA reductase inhibitors may be considered in the future as possible therapeutic agents for bone, a better understanding of the role of the cholesterol biosynthetic pathway in osteoblastic differentiation and function is needed.

ACKNOWLEDGMENTS

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